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Directing the immune system to attack tumors represents a potential powerful non-toxic approach for the treatment of breast cancer. Our goal is to ultimately engineer the interleukin-2 receptor (IL-2R) in cytotoxic T cells (CTL) to control signal transduction through this receptor and to improve the in vivo efficacy upon adoptive transfer to a tumor-bearing host. To this aim, we have continued to characterize a series of chimeric IL-2R constructs that depend upon drug-induced heterodimerization and prepare new constructs predicted to be more active as they are dependent upon homodimerization. We continue to refine our *in vivo* model of anti-tumor immunity. We show that naive CD8+ tumor-specific T cells very efficiently mediate anti-tumor responses independent of CD4+ helper cells.

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FOREWORD

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Introduction

IL-2 is an important cytokine that regulates a number of T cell activities, including promoting T cells growth, sensitizing T cells to activation-induced apoptosis, and enhancing CTL, LAK, and NK activity (1, 2). Early on, IL-2 showed much promise as an anti-tumor agent, but its use in patients has been problematic due to its short half-life and high toxicity in vivo. The major objective of this grant has been to develop an approach in which the value of IL-2 as a therapeutic agent could be carefully evaluated in model systems without this toxicity and short half-life. Our plan it to exploit small molecular weight drugs that function to dimerize signaling proteins by virtue of their specific binding to the FKBP-binding domain of immunophilin (3). This approach requires the molecular construction of chimeric proteins consisting of the receptor or signaling protein of interests and the FKBP domain. These type of proteins have been successful utilized to induce signaling by cells surface receptors, intracellular signaling proteins, or transcription factors (4-12). We proposed that this approach might be applied to the IL-2R since initiation of its signaling cascade first depends on dimerization of its β and γc subunits (13, 14).

Our research has maintained two parallel tracks. One has been the molecular construction and testing of chimeric IL-2R that places their signaling under the control of the small synthetic drug, AP1510. The second has been the development of a preclinical animal model that will allow us to evaluate the efficacy of such engineered IL-2R upon expression into tumor-specific T cells and their transfer to a tumor-bearing mouse.

Body (Results)

Development and characterization of chimeric receptors. In our last progress report, we described the construction and characterization of a series of chimeric receptors that contained the

cytoplasmic domains of IL-2R β or γc and one or more FKBP domains. These domains were placed at orientations that were at the amino or carboxyl terminus of the β or γc cytoplasmic domains. This was done because the size of the β or γc cytoplasmic domains substantially differ, and one of these constructs might yield a spatial orientation that promoted maximal dimerization by AP1510. When multiple FKBP domains were situated at the carboxyl region of β or γc cytoplasmic tail (designated pCMβF4E and pCMγF4E which both contain 4 FKBP domains), we detected some activation of STAT5 by AP1510 after transient transfection into COS7 cells with Jak1, Jak3 and STAT5a.

To test whether such constructs functioned in T cells, we used the EL4 thymoma that endogenously express normal levels of Jak1, Jak3 and STAT5s. Two pairs of chimeric IL-2R constructs were tested for STAT5 activation after stable transfection of these EL4 cells. One pair of constructs were identical to that mentioned above while another pair of constructs were similar, but these constructs encoded chimeric molecules that contained only 2 FKBP domains. As a positive control, we used the 1F1γWT cell line, which are the same EL4 cells but transfected with the native IL2Rαβγ.

In order to generate clones of EL4 which express both the β and γc chimeric constructs, we co-transfected EL4 cells with pZeo and pCMβF2E and isolated 1 zeocin resistant clone that expressed the chimeric β chain at a relatively high level as tested by Western blot analysis with antiserum to the epitope tag incorporated into the chimeric receptors. This clone was the further transfected with pCINeo and pCMγF2E, and zeocin and G418 drug resistant colonies were isolated that expressed both the β and γc constructs. In the same fashion, stable EL4 transfectants were generated that express both chimeric receptors encoded in pCMβF4E and pCMγF4E. A relatively high number of these transfected cells (3×10^7) were stimulated for 30 min with AP1510 or IL-2 for 1F1γWT. Cytoplasmic extracts were prepared and immunoprecipitated with anti-STAT5. Activation

of STAT5 was tested by Western blot analysis using anti-phospho-STAT5 (Fig. 1). Although STAT5 was highly activated in the 1F1γWT control cells by IL-2, there was no activation of STAT5 in any of these clones that expressed the chimeric IL-2R. The AP1510 was shown to be active by its capacity to induce proliferation in Baf cells transfected with a similarly designed chimeric c-kit receptor (7).

We believe it is important to note that when we analyzed these transfected Baf cells by Western blots, they expressed much higher levels of chimeric receptors than we detected for the transfected EL4. Therefore, there are 2 important practical concerns regarding the failure to activate IL-2R-like signaling by our chimeric molecules. First, the chimeric IL-2R transfected EL4 cells may not express the chimeric proteins in sufficient levels to induce IL-2R signals. Second, it has proved difficult to generate large number of transfected clones that will express both constructs in relatively equal and high levels so that there was a reasonable chance for biologically active heterodimerization. Additionally, and a theoretical concern that may be most critical, the FKBP domains in the chimeric β and γc constructs may not lie in proper orientation for efficient heterodimerization of these molecules by AP1510. In fact, in all the reported cases in which dimerization based on the FKBP domain was successful, the chimeric molecules showed biological activity due to homodimerization (4-12), and so far, there are no reported cases in which this approach has been successful for heterodimerization.

In order to overcome these problems, we initiated a companion approach in which the chimeric IL-2R were prepared to function as a single molecule. The notion here is to link the IL-2R β and the γc cytoplasmic domains in such that they will reside on the same molecule, so dimerization will lead to homodimers or homo-oligomers. As Jak-3 is the only known signaling molecule associated with γc, this cytoplasmic domain was linked to that of the β cytoplasmic domain or to the

β cytoplasmic domain that lacks the Jak-1 binding region. The reason for this latter construct is that it is predicted to precisely maintain the spatial relationship of the signaling domains to each other and the inner leaflet of the plasma membrane.

To test this idea, we constructed 2 sets of chimeric molecules (Fig. 2). Since the efficiency of AP1510 in our system is still unknown and the stability of the chimeric molecule with the FKBP binding sites is not clear, we designed the chimeric molecule with the extracellular and transmembrane domains of mouse c-kit upstream to the IL-2Rγ cytoplasmic tail followed by the cytoplasmic tail of IL-2Rβ, with and without its Jak-1 binding region. These construct were named c-kitγβ and c-kitβγAH, respectively, and these were cloned into pCINeo under the control of CMV promoter. The c-kit chimeric constructs have been analyzed by sequencing and their expression was tested after transient transfection into COS-7 cells. The transfected cells were subjected to FACS analysis with anti-c-kit as well as Western blot analysis with anti-IL-2Rβ or anti-γc antisera (Fig. 3). This latter analysis demonstrated that the transfected COS7 cells expressed the appropriate chimeric proteins. For the second set of constructs, similar chimeric receptors, as shown in Fig. 2, that depend upon FKBP and AP1510 rather than c-kit and Stem cell factor (SCF) have been prepared and are currently being verified by DNA sequence analysis.

To test the biological activity of these new chimeric IL-2R, the IL-2-dependent CTLL and EL4 cells were transfected with the c-kit constructs. At this juncture, 50 and 40 drug-resistant CTLL and 28 and 30 drug-resistant EL4 cells have been isolated that were transfected with ckitγβ or ckitγβ(AH), respectively. These clones were screened for the level of expression of the chimeric constructs by Western or FACS analysis. Fig.4 illustrates that the expression of the chimeric c-kit protein was expressed by some of the transfected CTLL cells as assessed by Western blot analysis using anti-IL-2Rβ antisera. This analysis also provides a direct comparison between the levels of

expression of endogenous and the chimeric IL-2Rβ. The CTLL clones which express relatively high levels of the chimeric molecules are currently being analyzed for proliferation in the presence of SCF without IL2. The EL4 clones which expressed relatively high levels of the chimeric molecules will be analyzed for STAT5 activation in the presence of SCF.

In vivo model for T cell anti-tumor reactivity. In the previous progress report, we showed that a tumor immune response could not be elicited in tumor-bearing mice after the adoptive transfer of a relatively high number of naive transgenic tumor specific CD8+ T cells. The transgenic T cells are designated OT-1 and are specific for ovalbumin (OVA), which was used as a model tumor-specific antigen that was expressed in the E.G7 tumor cell line (15, 16). Importantly, as assessed at several different time points after adoptive transfer, the tumor-specific transgenic T cells were not activated to proliferate in both the spleen and the draining lymph nodes in the presence of the growing solid tumor. The failure to activate the T cells was not because the tumor anergized the T cells since OT-I T cells derived from tumor-bearing mice readily proliferated upon antigen challenge in vitro. In contrast, adoptive transfer of pre-activated OT-I CTL inhibited tumor growth in a dose-dependent manner indicating that E.G7 was susceptible to immune effector cells. Importantly, naive OT-I T cells proliferated in the mice only if they were adoptively transferred together with GM-CSF-induced bone marrow derived OVA-pulsed APC.

We have continued to work during the last year to refine this model. We tested whether the in vivo induction of OT-I effector cells also induced anti-tumor immunity. Naive OT-I T cells were adoptively transferred to mice 5 days after s.c. injection of E.G7, and 24 hr later the mice received OVA-pulsed bone marrow derived APC. As shown earlier, the tumor grew quickly in mice that received tumor and only naive OT-1 T cells (Fig. 5A). By contrast, co-administration of OT-I and OVA-pulsed APC resulted in a substantial delay in the progression of the tumor. In 40% of the mice,

no tumor was detected 45 days after injection of E.G7, and several mice, that were observed longer, remained tumor-free on day 60. Importantly, there was no inhibition of growth when tumor-bearing mice received OVA-pulsed APC in the absence of OT-I T cells. This result indicates that the inhibition of tumor growth is dependent upon the presence of the adoptively transferred transgenic T cells. Thus, after appropriate activation either in vitro or in vivo, E.G7 was susceptible to tumor (OVA)-specific OT-I effector cells.

The E.G7 tumor eventually grew in all mice that received in vitro induced OT-I effector cells and in some mice that were stimulated with OVA-pulsed APC in vivo. The E.G7 cells were excised from one such mouse in each of the treatment groups and grown in culture for at least 7 days. These cells were then used as targets for OT-I CTL generated by in vitro culture. The E.G7 cells obtained from the mice treated with ex vivo induced OT-I were nearly as good targets for OVA-specific CTL as the parental E.G7 (Fig. 5B). Thus, the tumor outgrowth from this mouse appears to be the result of a failure of the adoptively transferred CTL. However, E.G7 from the in vivo APC-treated mice were not lysed by the OVA-specific CTL, suggesting that tumor outgrowth in this case was caused by the selection of a tumor variant that escaped the effector OT-I CTL. In addition, ELISA analysis confirmed that these cells failed to secrete detectable OVA (data not shown). Collectively, these data raise the possibility that in vivo induced effector T cells induced a more potent anti-tumor immune response than adoptive T cell immunotherapy.

In addition, we tested whether this anti-tumor response depends upon help from CD4 T cells. To that end we used CD4 knock-out mice to conduct the same experiment as described above. The mice received 1 x 10^6 E.G7 cells and 5 days later received 2.5 x 10^6 OT-I cells. One day later, some mice received OVA-pulsed APC. The results showed that, like normal B6 mice, the CD4 knockout mice elicit an effective anti-tumor response, if they were adoptively transferred with OT-I and OVA-

pulsed APC.(Fig. 6C). As expected, adoptive transfer of only OT-I did not elicit an anti-tumor response. These data, therefore, directly demonstrate that CD4 T cell help is not required for this anti-tumor response.

Key research accomplishments

- A mouse model of tumor immunity has been developed in which a defined antigen that marked a solid tumor was rejected in a dose-dependent manner by the adoptive transfer of T cell receptor transgenic CD8 effector cells specific to this model tumor antigen or by immunization of tumor-bearing mice containing naive transgenic T cells with antigen-pulsed APC
- Mice adoptively transferred with relatively large number of naive tumor-specific transgenic T cells do not affect the growth of a solid subcutaneous tumor. This failed response was due to immunological ignorance, not an aborted immune response.
- Induction of an anti-tumor immune response by the CD8+ T cells occurred without CD4+ helper T cells.

Reportable outcomes

Dalyot-Herman, N, and Malek, T. R. Anti-tumor immunity of naive and activated class-I-restricted tumor-specific TCR-transgenic T cells (Abstract) Experimental Biology 1999.

Dalyot-Herman, N. and Malek, T.. R. Reversal of T cell ignorance and induction of anti-tumor immunity by peptide-pulsed antigen presenting cells (Abstract) Era of Hope DOD Meeting, 2000.

Dalyot-Herman, N., Bathe, O. and Malek, T. R. Reversal of CD8+ T cell ignorance and induction of anti-tumor immunity by peptide-pulsed antigen presenting cells (submitted to *J. Immunol*; currently under revision).

Conclusions

Based on our past success with chimeric CD8/IL-2R and those reported by others (13, 14), we still conclude that the development of a drug-regulated chimeric IL-2R is still a technically achievable goal. Our past capacity to detect activation of chimeric IL-2R with the FKBP domain further supports this contention. However, from these data and the inability to detect functional chimeric IL-2R in the EL4 T cells suggest several limitations for this approach. First, it is likely that dimerization of chimeric receptors is less efficient than dimerization of native IL-2R by IL-2. In our one observed success, the level of activation of STAT 5 in the transfected COS7 was still lower than seen with IL-2R transfected COS7 even though the COS7 transfected with the chimeric IL-2R expressed very high levels of these proteins. The expression of such chimeric proteins were substantially lower in the transfected EL4, providing one plausible explanation for the inability to detect induction of STAT5 phosphorylation by AP1510. Second, for AP1510 to induce heterodimerization, the FKBP domains on the chimeric molecules must be in sufficient proximity to allow binding of both the IL-2Rβ and γc chimeric proteins by AP1510. Unfortunately, there is no way to specifically verify this, so it must be empirically tested.

Our current approach, as outlined above, avoids these problems as the development of chimeric IL-2R now depends on homodimerization or multimerization. By design, this approach should permit more rapid development of transfected T cells to test the biological activity of these molecules. Already, we have generated in a much more rapid fashion a number of EL4 and CTLL T

cells that express the chimeric c-kit/IL-2R. Thus, we should be able to prepare transfected cells that will vary in the levels of the chimeric protein and compare the resulting functional activity with the levels of endogenous IL-2R. By developing analogous c-kit and FKBP chimeric IL-2R, we also will be able to directly relate the relative effectiveness of IL-2 vs SCF vs AP1510, which is critical to ultimately apply AP1510 to a therapeutic protocol.

We have established an animal model to test the efficacy of these chimeric constructs. From these experiments we have learned several important issues with respect the utility of tumor-specific CTL in immunotherapy. First, naive T cells, even in high numbers, are ignorant of a solid subcutaneous tumor. The notion that immunological ignorance is an important mechanism that prevents anti-tumor immunity is not widely accepted and has only been occasionally suggested to account for failed anti-tumor responses (17-20). These naive T cells, however, can be induced in vivo to mount an anti-tumor response that is independent of CD4 T cell help. This latter issue is significant because it demonstrates the potential to treat tumor patients direct with only CD8+ T cells. Second, the adoptive transfer of in vitro generated CTL results in an anti-tumor effect, but such an effect is limited and not simply due to tumor antigen escape variants. An important issue that we wish to address is whether the anti-tumor response by in vitro CTL are limited based on the survival of the transferred CTL, as this is predict to improve by stimulation of the IL-2R in vivo.

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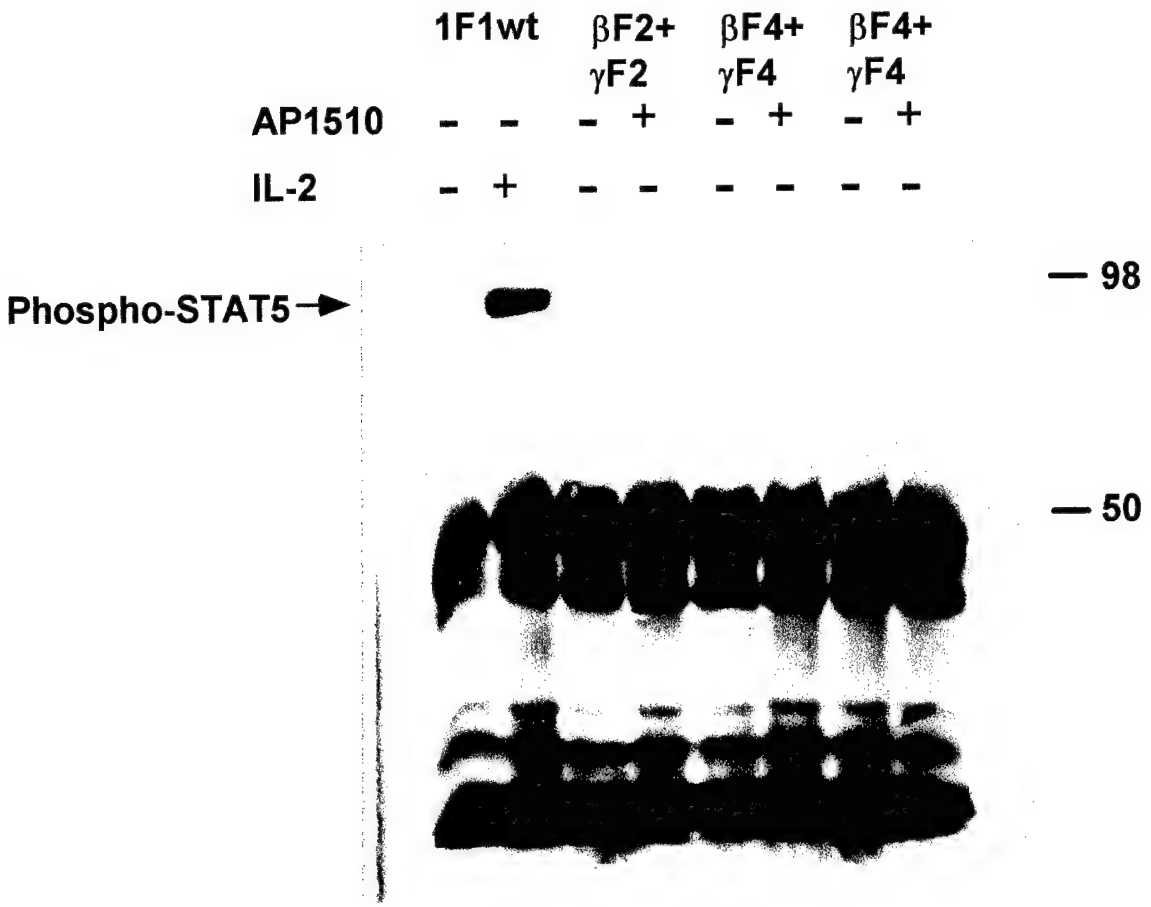

Fig. 1: Ability of the AP1510 to induce phosphorylation of STAT5a in stable clones of EL4 cells transfected with the chimeric molecules. 3×10^7 cells of each clone (as indicated on top) were collected and induced for 30 min. at 37°C with IL-2 or AP1510. Protein extracts were harvested and immunoprecipitated with anti-STAT5. Western blot hybridization was performed with anti-phospho-STAT5.

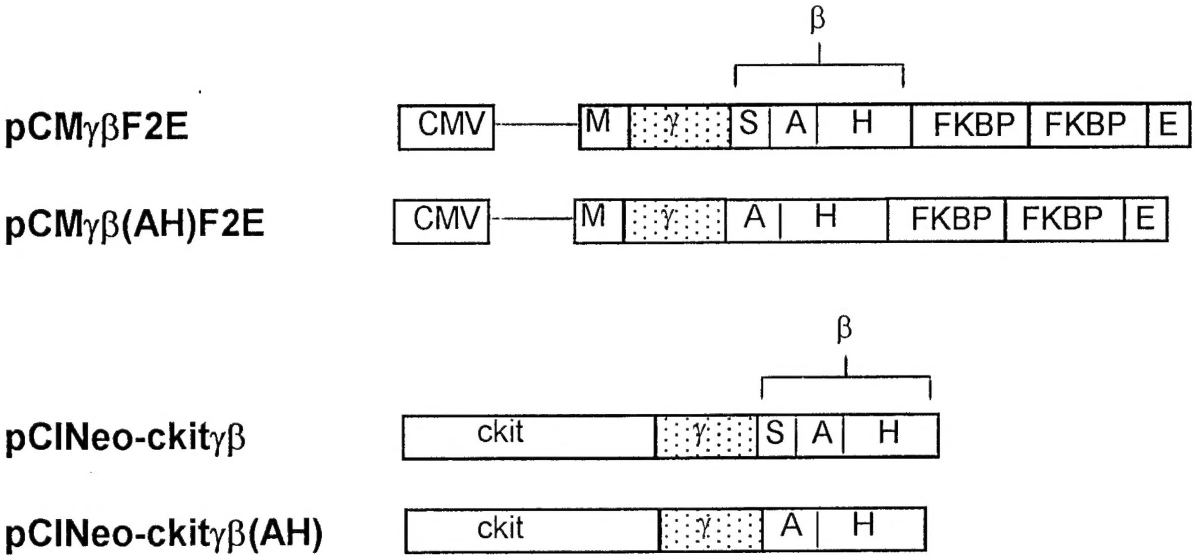

Fig. 2: Constructs of IL-2R chimeric molecules.
pCMγβF2E and pCMγβ(AH)F2E were constructed by inserting the PCR fragments of IL-2Rβ (S-A-H or A-H domaines, respectively) into the IL-2Rγ cytoplamic tail at the AvrII site using the SpeI site.
CMV,CMV promoter; M- Myristoylation signal; FKBP, binding domain for FKBP12 or AP1510; β- cytoplasmic tail of IL-2Rβ, γ, cytoplasmic tail of IL-2Rγc; E, HA epitope.
pCINeo-CKITγβ and pCINeo-CKITγβ(AH) were constructed by inserting PCR fragments of the extracellular and transmembrane domaine of CKIT (XhoI-EcoRI), IL-2Rγ cytoplamic tail (EcoRI-SalI) and IL-2Rβ cytoplamic tail (S-A-H or A-H domaines, respectively) (SalI-NotI) into the multiple cloning sites of pCINeo plasmid.

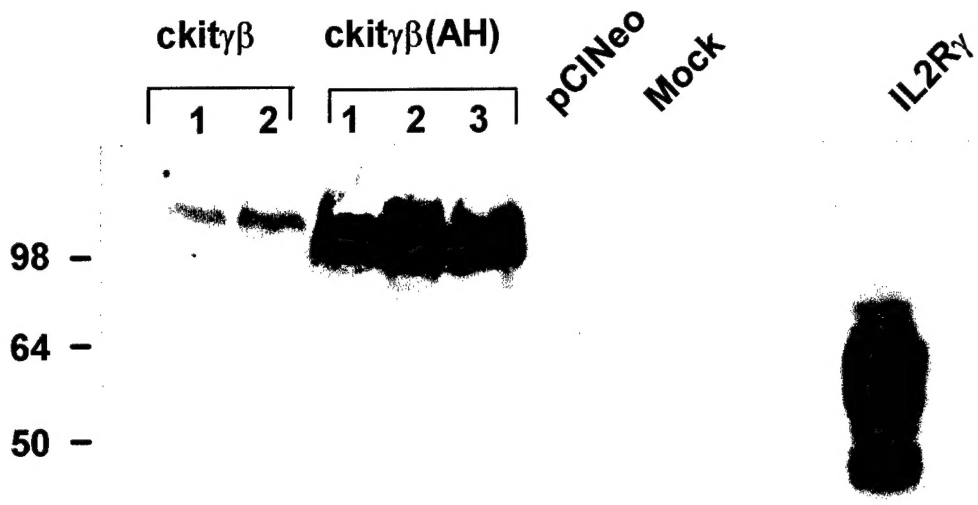

Fig 3: Expression of the chimeric constructs in COS-7 cells.

3 x 10^6 cells were transfected by electroporation with 2μg of each plasmid. (the numbers represent different plasmid harvested from different bacteria transformants). 3 days later the cells were collected and protein extracts were harvested and subjected to Western blot hybridization with anti-IL2Rγ. For positive control, cells were transfected with the plasmid carrying the native IL2Rγ. As a negative control, cells were transfected with the empty plasmid vector pCINeo or without DNA, Mock, as indicated.

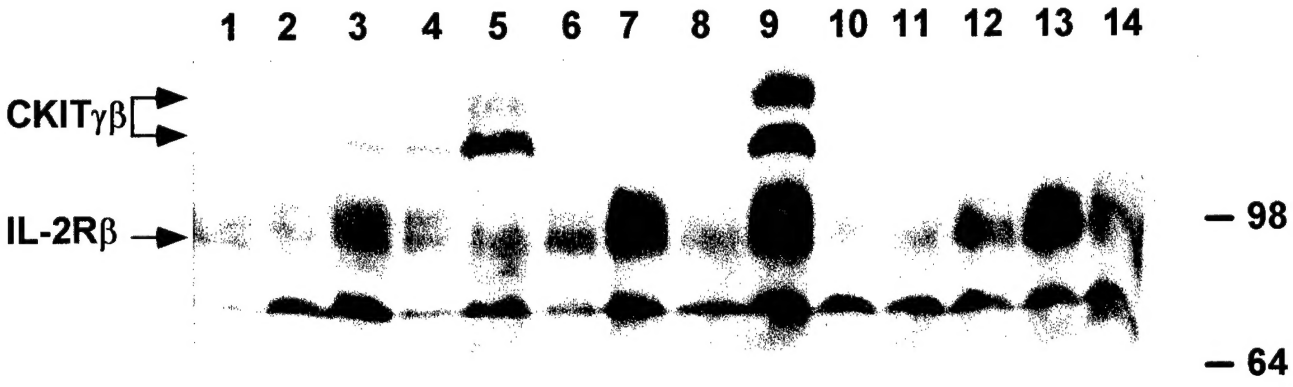

Fig. 4: Expression levels of endogenous IL-2Rβ and chimeric cKITγβ constructs in stably transfected CTLL cells.

3-5 x 10^6 cells of stably transfected CTLL clones were collected and protein extracts were harvested and subjected to Western blot analysis with anti-IL2Rβ. The numbers on top represent the different clones and the arrows indicate the expected protein bands.

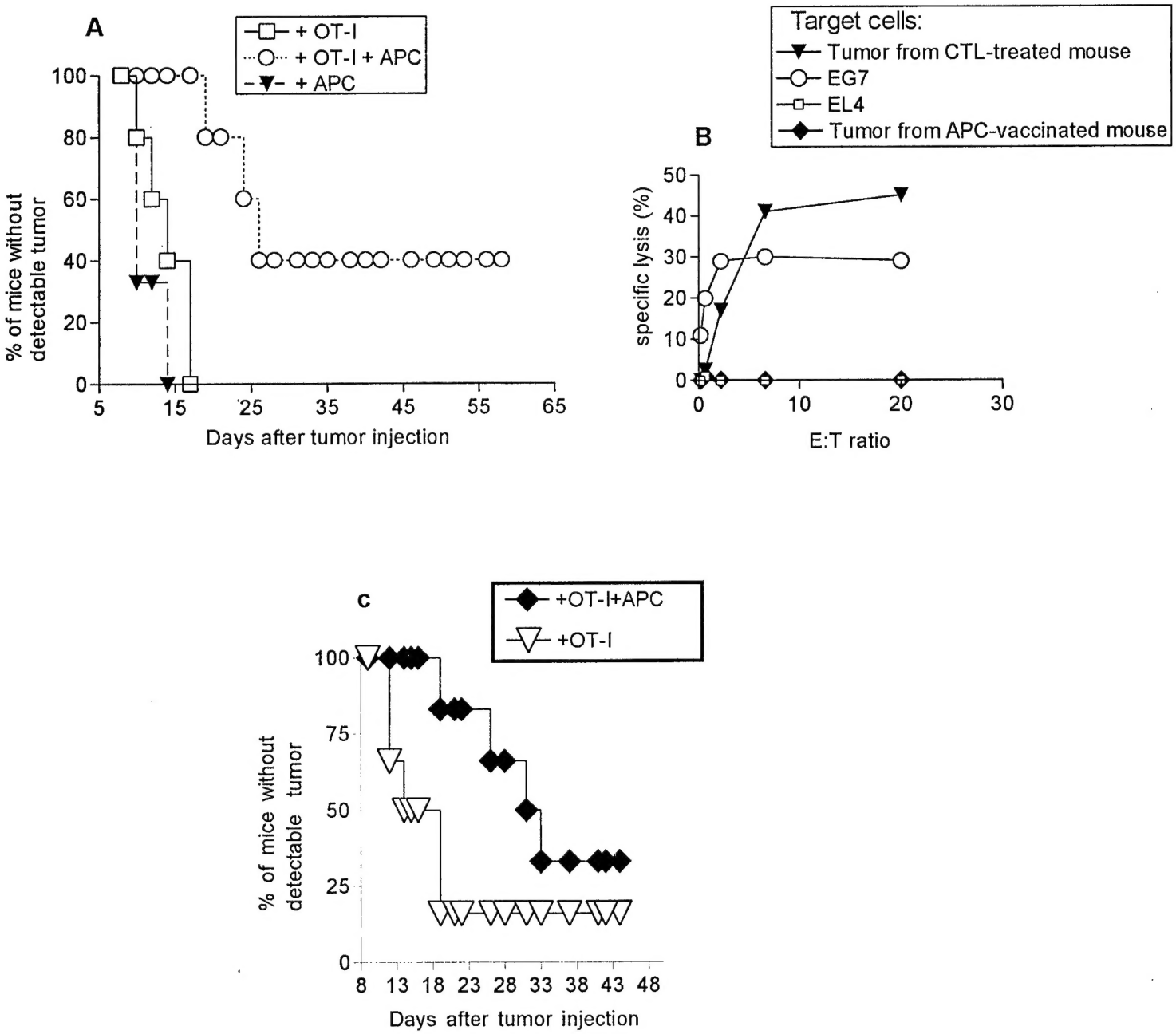

Fig. 5: Anti-tumor responses by naive OT-I tumor-specific T cells. A) Normal C57BL/6 mice or C) CD4 knockout mice were injected with E.G7 (1 x 10^6). Five days later mice received naive OT-I T cell (3 x 10^6) and 1 day later OVA peptide-pulsed APC (0.5 x 10^6), as indicated. The size of the tumor was recorded. Data shown contain 5-6 mice/group. B) Lysis of tumor cells from OT-I-treated mice by OT-I effector cells in vitro. Tumors were excised from treated mice, as designated, grown in culture for at least 7 days and were labeled with ^{51}Cr to serve as targets for OT-I CTL. ^{51}Cr-labeled E.G7 and EL4 served as positive and negative control targets, respectively.